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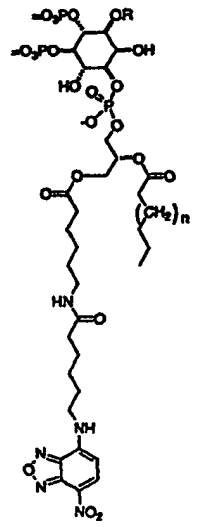
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(54) Title: DELIVERY OF PHOSPHOINOSITIDE POLYPHOSPHATES INTO CELLS

(57) Abstract

A method for facilitating delivery of a phosphatidylinositol polyphosphate or derivative thereof into a eukaryotic cell is disclosed. The method includes forming a complex of the phosphatidylinositol polyphosphate or derivative with a polyamine, and then contacting the cell with the complex. Preferred polyamines include aminoglycosides, dendrimeric polyamines, and histones. Compositions of matter for use in the method are also described. A method for screening compounds for minimum toxicity to eukaryotic cells and maximum toxicity to bacterial cells is also disclosed. Also disclosed is a method for monitoring calcium flux in a cell.



R =H, PtdIns(4,5)P_2-NBD
C_8 (n = 1) and C_{16} (n = 13)

R =PO_3=,PtdIns(3,4,5)P_3-NBD
C_{16} (n = 11)

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DELIVERY OF PHOSPHOINOSITIDE POLYPHOSPHATES INTO CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/102,482, filed September 30, 1998.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under Grant No. NS-29632 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

This invention relates to compositions and methods for delivery of phosphatidylinositol polyphosphates (phosphoinositides, PIP_ns) and inositol polyphosphates (IP_ns) into cells of both eukaryotes and prokaryotes. Included among these cells are cells of animals, including parasites such as protozoa and helminths, plants, fungi, and bacteria. More particularly, the invention relates to delivery of PIP_ns, IP_ns, and derivatives thereof into cells using polyamine shuttles.

Phosphatidylinositol polyphosphates serve as signaling molecules in numerous and diverse eukaryotic cellular processes. T.F.J. Martin, Phosphoinositide lipids as signaling molecules: Common themes for signal transduction, cytoskeletal regulation, and membrane trafficking. 14 Annu. Rev. Cell Dev. Biol. 231-264 (1998); A. Toker, The synthesis and cellular roles of phosphatidylinositol 4,5-bisphosphate. 10 Curr. Opin. Cell Biol. 254-261 (1998); A. Toker & L.C. Cantley, Signaling through the lipid products of phosphoinositide-3-OH kinase. 387 Nature 673-676 (1997). They are essential elements in tyrosine kinase growth factor receptor and G-protein receptor signaling pathways. B. Stoyanov et al., Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase, 269 Science 690-693 (1995); L.R. Stephens et al., The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101, 89 Cell 105-114 (1997). Furthermore, they have important roles in endocytic, exocytic, and Golgi vesicle movement, P. De Camilli

et al., Phosphoinositides as regulators in membrane traffic, 271 Science 1533-1539 (1996), and remodeling of the actin cytoskeleton, A. Hall, Rho GTPases and the actin cytoskeleton, 279 Science 509-514 (1998). A detailed understanding of the mechanisms by which phosphoinositides elicit their effects is limited by the current inability to monitor changes in cellular localization as the physiology of the cell is altered.

Activation of cellular signaling pathways can result from specific changes in membrane phosphoinositide phosphorylation in response to stimuli. The number and location of phosphate monoesters on the inositol head group are controlled by specific kinases and phosphatases and determines the role of each PIP_n. A. Toker, The synthesis and cellular roles of phosphatidylinositol 4,5-bisphosphate, 10 Curr. Opin. Cell Biol. 254-261 (1998); A. Toker, & L.C. Cantley, Signaling through the lipid products of phosphoinositide-3-OH kinase, 387 Nature 673-676 (1997). Thus, different PIP_ns have specific roles in signaling pathways, cytoskeletal architecture, T.F.J. Martin, Phosphoinositide lipids as signaling molecules: Common themes for signal transduction, cytoskeletal regulation, and membrane trafficking, 14, Annu. Rev. Cell Dev. Biol. 231-264 (1998); A. Hall, Rho GTPases and the actin cytoskeleton, 279 Science 509-514 (1998), or membrane trafficking for a given cell type, P. De Camilli et al., Phosphoinositides as regulators in membrane traffic, 271 Science 1533-9 (1996). Phosphoinositide composition is dynamic in time and space, and to locate specific signaling events, it is necessary to devise a technique to study the localization of newly-synthesized PIP_ns in cellular membrane domains. An attractive approach would be to have a cell-permeant form of any given PIP_n, which would permit direct observation of PIP_n changes and redistribution in specific cellular compartments following activation by extracellular signals.

Investigators have sought cell-permeant derivatives that would allow delivery of the active PIP_ns or IP_ns intracellularly without disruption of the cell membrane. To date, several compounds have been prepared as lipid-soluble analogs of inositol trisphosphates [$Ins(1,3,4)P_3$, $Ins(1,4,5)P_3$] and phosphoinositides [$PtdIns(3,4,5)P_3$]. T. Jiang et al., Membrane-permeant esters of phosphatidylinositol 3,4,5-trisphosphate, 273 J. Biol. Chem. 11017-24 (1998); W.H. Li et al., Membrane-permeant esters of inositol polyphosphates, chemical syntheses and biological applications, 53 Tetrahedron 12017-12040 (1997); M.T. Rudolf et al., A membrane-permeant, bioactivatable derivative of $Ins(1,3,4)P_3$ and its effect on Cl- secretion from T-84 cells, 8 BioMed. Chem. Lett. 1857-1860 (1998). In addition,

"caged" Ins(1,3,4,5)P_4, Ins(1,4,5)P_3, and $InSP_6$ have been synthesized and employed in cellular studies. W.H. Li et al., Cell-permeant caged InsP(3) ester shows that Ca^{2+} spike frequency can optimize gene expression, 392 Nature 936-941 (1998); J. Chen & G.D. Prestwich, Regioselective synthesis of photolabile P(1,2)- and P(4,5)-(*O*-nitrobenzyl) esters of *myo*-inositol 1,2,3,4,5,6-hexakisphosphate, 38 Tetrahedron Lett. 969-972 (1997). Preparation of each of these analogs has involved extensive chemical synthesis, W.H. Li et al., Membrane-permeant esters of inositol polyphosphates, chemical syntheses and biological applications, 53 Tetrahedron 12017-12040 (1997). Moreover, subsequent deprotection (= activation) by light and/or esterase action has been required to release the free IP_n or PIP_n. Thus, the photolabile "caged" analogs require UV irradiation to release the anionic phosphates, while acetoxymethyl phosphate esters and butyrate esters of the ring hydroxyls require cellular esterases for activation. An insurmountable difficulty with these elegant techniques is that multiple protecting groups are attached to each cell-permeant molecule. For example, seven protecting groups with differing lability are attached to the cell-permeant Ptdlns(3,4,5)P_3. W.H. Li et al., Cell-permeant caged InsP(3) ester shows that Ca^{2+} spike frequency can optimize gene expression, 392 Nature 936-941 (1998). With the exception of the photolabile groups, an investigator has little control over the rate of release of the active material in the cell. More importantly, a heterogeneous mixture of molecular species is produced following stepwise intracellular deprotection, and thus the true agonists may remain ambiguous and cryptic.

U.S. Patent No. 5,783,662 to P.A. Janmey et al. describes covalent conjugates of N-terminal-blocked polyphosphoinositide binding peptides for use in facilitating transport of a membrane-impermeable extracellular agent across the membrane of the cell. The extracellular agent, such as a peptide, oligonucleotide, or antibiotic, is covalently coupled to the carboxyl terminus of a transport mediating peptide to form a prodrug having the formula X-P-A, wherein X is an amine derivatizing agent, P is a polybasic transport-mediating peptide, and A is the extracellular agent, which is transported across the cell membrane. In an embodiment of the invention, X is a fluorescent molecule, such as rhodamine or fluorescein, and P is a peptide domain of human gelsolin.

U.S. Patent No. 5,693,521 to R.Y. Tsien & C. Schultz discloses acyloxyalkyl esters of phosphate-containing second messengers, such as cAMP, cGMP, inositol triphosphate, and inositol tetraphosphate, which are capable of permeating cell membranes. Once inside the

cell, the ester derivatives undergo enzymatic conversion to the biologically active form of the second messenger.

WO 98/15629 by M.P. Czech & J.K. Klarlund discloses binding proteins for phosphatidylinositides. These binding proteins, referred to as "general receptors for phosphoinositides" or "GRPs"exhibit a functionally and structurally modular form that includes a subdomain homologous to the yeast SEC7 gene product and a pleckstrin homology (PH) domain. GRP proteins preferably exhibit high affinity binding to products of the lipid kinase, phosphatidylinositol-3-OH kinase (i.e., PI 3-kinase), e.g., phosphatidylinositol-3,4,5-trisphosphate. By modulating GRP activity, cell adhesion, membrane trafficking, insulin action on glucose transport of a cell, or nucleotide exchange of small GTP-binding proteins may be modulated. GRP proteins may also be used for screening test compounds for modulators of an interaction between a PI 3-kinase product and a GRP polypeptide receptor.

WO 97/46688 by B. Vanhasebroeck & M.D. Waterfield discloses the lipid kinase, p110δ, which is part of the PI 3-kinase family. The PI 3-kinase p110δ interacts with p85, has a broad phosphoinositide specificity, and is sensitive to the same kinase inhibitors as PI 3-kinase 110α. In contrast to previously known PI 3-kinases, which show a ubiquitous pattern of expression, p110δ is selectively expressed in leukocytes. Further, p110δ shows enhanced expression in most melanomas tested and, therefore, may play a role in regulating the metastatic property exhibited by melanomas.

The publications and other reference materials referred to herein to describe the background of the invention and to provide additional detail regarding its practice are hereby incorporated by reference. The references discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

In view of the foregoing, it will be appreciated that providing compositions and methods for delivery into cells of phosphoinositides and their chemical derivatives would be a significant advancement in the art.

BRIEF SUMMARY OF THE INVENTION

It is an object of the present invention to provide compositions and methods for delivery of phosphoinositides into cells.

Another object of the invention is to provide compositions and methods for visualizing the location of fluorescently labeled phosphoinositides during changes in cell physiology.

Still another object of the invention is to provide a method for screening inhibitors of changes in cell physiology.

Yet another object of the invention is to visualize the uptake and localization of aminoglycosides.

A still further object of the invention is to develop antibiotic selectivity assays by observing selective uptake of fluorescent aminoglycosides in pathogens versus human or animal cells.

It is also an object of the invention to identify new therapeutic compounds that can affect the selectivity of aminoglycoside uptake.

Another object of the invention is to provide a high-throughput screen for natural products and synthetic compounds that affect cellular uptake and targeting of both aminoglycosides and phosphoinositides to cells from vertebrate tissues versus cells of pathogenic organisms.

Still another object of the invention is to use PIP_n-fluorescent aminoglycoside uptake for the visualization of uptake and subcellular localization of aminoglycosides.

Yet another object of the invention is to develop antibiotic selectivity assays by observing selective uptake of fluorescent aminoglycosides in pathogens, including bacteria, protozoal parasites, and the like, versus human or animal cells.

These and other objects can be addressed by providing a composition of matter comprising a mixture of a phosphoinositide polyphosphate, inositol polyphosphate, or mixture thereof and a polyamine. Preferably, each of the phosphoinositide polyphosphate, inositol polyphosphate, or mixture thereof and polyamine is labeled with a detectable label. Preferred labels include fluorescent labels, radiolabels, chemiluminescent labels, spin labels, photophores, chromophores, biotin, nanogold particles, and the like, and mixtures thereof. Fluorescent labels are especially preferred. Preferred polyamines include aminoglycosidic aminocyclitols (e.g., aminoglycoside antibiotics), dendrimeric polyamines, histones, polybasic polypeptides, lipidic polyamines, polyethyleneimine, steroidal polyamines, and the like, and mixtures thereof.

A method for facilitating uptake of a phosphoinositide polyphosphate, inositol polyphosphate, or mixture thereof into a cell comprises contacting the cell with a composition of matter comprising a mixture of the phosphoinositide polyphosphate, inositol polyphosphate, or mixture thereof and a shuttle. In a preferred embodiment of the invention, such a shuttle is a polyamine such as aminoglycosidic aminocyclitols (e.g., aminoglycoside antibiotics), dendrimeric polyamines, histones, polybasic polypeptides, lipidic polyamines, polyethyleneimine, steroidal polyamines, and the like, and mixtures thereof.

In another aspect of the invention, a method for facilitating uptake of an aminoglycoside antibiotic into a cell comprises contacting the cell with a composition of matter comprising a mixture of (a) a phosphoinositide polyphosphate, inositol polyphosphate, or mixture thereof, and (b) the aminoglycoside antibiotic. Preferably, the cell is a pathogenic cell, such a prokaryotic cell or cell of a parasite, such as a protozoal cell, helminthic cell, or fungal cell.

In another aspect of the invention, a method for visualizing the uptake and localization of an aminoglycoside in a cell comprises:

(a) mixing an aminoglycoside bearing a visually identifiable label with a polyamine to result in a mixture;

(b) contacting the cell with the mixture such that the mixture is delivered into the cell; and

(c) observing the label, thereby visualizing the uptake and localization of the aminoglycoside in the cell.

In this method, the cell can be a eukaryotic cell, such as an animal, plant, protozoal, helminthic, or fungal cell. The cell could also be a prokaryotic cell.

In still another aspect of the invention, a method for screening for a compound that minimizes cytotoxicity of aminoglycoside antibiotics to mammalian cells comprises:

(a) mixing an aminoglycoside bearing a label with a polyamine to result in a mixture;

(b) contacting a mammalian cell with the mixture and with a compound to be tested such that, absent the presence of the compound, the mixture would be delivered into the cell; and

(c) observing the label, thereby observing the uptake and localization of the aminoglycoside in the mammalian cell, wherein reduced uptake of the aminoglycoside or

altered localization of the aminoglycoside as compared to the cell treated with the mixture in the absence of the compound indicates reduced cytotoxicity.

A further aspect of this method comprises determining whether uptake of the aminoglycoside into a bacterial cell is substantially unaltered in the presence of the compound.

In yet another aspect of the invention, a composition of matter comprises an aminoglycoside antibiotic covalently bonded to a fluorescent compound. Preferably, the aminoglycoside antibiotic is covalently bonded to the fluorescent compound through a linker moiety, such as an isothiocyanate or succinimidyl ester group. In especially preferred embodiments of this invention, the aminoglycoside antibiotic is neomycin and the fluorescent compound is rhodamine B or XR.

In a still further aspect of the invention, a method for monitoring calcium flux in a cell comprises:

(a) loading the cell with a calcium indicator, exchanging the medium, and monitoring the cell until no intensity change in the calcium indicator is observed;

(b) then contacting the cell with a complex of Ins(1,4,5)P_3 and a shuttle such that the Ins(1,4,5)P_3 enters the cell and thereby modulates the calcium flux thereof; and

(c) detecting the calcium indicator wherein a change in the intensity of the calcium indicator indicates a change in calcium flux in the cell.

Preferably, the calcium indicator is calcium crimson or Fluo-3 and the change in intensity is detected as a change in fluorescence.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

FIGS. 1a-d show the structures of fluorescently labeled phosphoinositides, inositol polyphosphates, and aminoglycosides according to the present invention. FIG. 1a shows the structure of PtdIns(4,5)P_2-NBD and PtdIns(3,4,5)P_3-NBD analogs with C_{18} (n = 13), C_{16} (n = 11), and C_6 (n = 1) *sn*-2-*O*-acyl chain lengths. FIG. 1b shows the structure of Ins(1,4,5)P_3-XRITC, a fluorescent analog of IP_3. FIGS. 1c-d show structures of two fluorescent neomycin derivatives, Neomycin-XRITC (Neo-XR) and Neomycin-Rhodamine B (Neo-RB), respectively; the open arrows indicate that either of the two primary aminomethyl substituents could bear the thiourea-linked fluorophore.

FIGS. 2a-c show delivery by polyamines of fluorescently labeled phosphoinositides and inositol polyphosphates into mammalian cells. FIG. 2a shows NIH 3T3 mouse fibroblast

cells with internalized PtdIns(4,5)P_2-NBD/histone; in the no-carrier (histone) control shown in the inset, the PtdIns(4,5)P_2-NBD fluorescence is extracellular. FIG. 2b shows Ins(1,4,5)P_3 was delivered to NIH 3T3 cells using histone protein as a carrier; in the inset, no histone was added to the Ins(1,4,5)P_3, and essentially all of the Ins(1,4,5)P_3 fluorescence is extracellular. In FIG. 2c, PtdIns(3,4,5)P_3-NBD was delivered into NIH 3T3 fibroblast cells using histone as a shuttle. PtdIns(3,4,5)P_3-NBD was mixed with histone and added to NIH 3T3 mouse fibroblast cells grown on coverslips. Low magnification (FIG. 2c) and high magnification (FIG. 2c inset) images were collected 10 minutes following addition of the PtdIns(3,4,5)P_3 derivative. The PtdIns(3,4,5)P_3-NBD localized to the plasma membrane, nuclear envelope, and punctate cytosolic regions coincident with ER and Golgi staining. Images were collected 30 minutes (FIG. 2a inset and FIG. 2b inset), 5 minutes (FIG. 2a), or 10 minutes (FIG. 2b and FIG. 2c) after dye solutions were added to the cells. The micrographs were obtained with a laser scanning confocal microscope. The bright cellular structures are due to the fluorescence of the PtdIns(4,5)P_2-NBD (FIG. 2a); Ins(1,4,5)P_3-XRITC (FIG. 2b); or PtdIns(3,4,5)P_3-NBD (FIG. 2c).

FIGS. 3a-b show phosphoinositide-mediated delivery of neomycin-rhodamine into NIH 3T3 cells. Neomycin-rhodamine was added to the medium of NIH 3T3 cells attached to cover slips, but remained almost exclusively extracellular for more than 10 minutes, as observed in FIG. 3a wherein the bright extracellular medium results from the neomycin-rhodamine fluorescence. In contrast, when neomycin-rhodamine was mixed with PtdIns(4,5)P_2 prior to being added to the medium, the neomycin-rhodamine rapidly accumulated in the cells as observed in FIG. 3b wherein the fluorescence from the neomycin-rhodamine is concentrated in subcellular structures. The micrographs were obtained with a laser scanning confocal microscope 10 minutes after addition of the neomycin-rhodamine or neomycin-rhodamine/PtdIns(4,5)P_2 complex.

FIGS. 4a-d show delivery of neomycin and phosphoinositides into prokaryotic and non-mammalian eukaryotic cells. In FIG. 4a, a fluorescent neomycin (FITC-labeled) was delivered into *E. coli* cells as a complex with PtdIns(4,5)P_2. In FIG. 4b, PtdIns(4,5)P_2-NBD was delivered to *E. coli* cells as a complex with histone protein. In FIG. 4c, a neomycin-rhodamine/PtdIns(4,5)P_2 complex entered the protozoan pathogen, *Cryptosporidium parvum* (bright spots among the field of epithelial cells). In FIG. 4d, a PtdIns(4,5)P_2-NBD/histone complex rapidly entered *C. parvum*. The micrographs were obtained with a laser scanning

confocal microscope 5 minutes after addition of the neomycin-FITC/PtdIns(4,5)P_2; PtdIns(4,5)P_2-NBD/histone; or neomycin-rhodamine/PtdIns(4,5)P_2 complexes to the cell media. The *C. parvum* cells were cultured on cover slips coated with bovine epithelial cells. In FIGS. 4a-d, the bright fluorescence is from the fluorophores (FITC, NBD, rhodamine) attached to neomycin (FIGS. 4a and 4c) or PtdIns(4,5)P_2 (FIGS. 4b and 4d).

DETAILED DESCRIPTION

Before the present compositions and methods for delivery of phosphoinositides and inositol polyphosphates into cells are disclosed and described, it is to be understood that this invention is not limited to the particular configurations, process steps, and materials disclosed herein as such configurations, process steps, and materials may vary somewhat. It is also to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

It must be noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. For example, reference to "a phosphoinositide polyphosphate" includes reference to two or more of such phosphoinositide polyphosphates, reference to "an inositol polyphosphate" includes reference to two or more of such inositol polyphosphates, and reference to "an aminoglycoside" includes reference to two or more of such aminoglycosides.

In describing and claiming the present invention, the following terminology will be used in accordance with the definitions set out herein.

As used herein, "comprising," "including," "containing," "characterized by," and grammatical equivalents thereof are inclusive or open-ended terms that do not exclude additional, unrecited elements or method steps. "Comprising" is to be interpreted as including the more restrictive terms "consisting of" and "consisting essentially of."

As used herein, "consisting of" and grammatical equivalents thereof exclude any element, step, or ingredient not specified in the claim.

As used herein, "consisting essentially of" and grammatical equivalents thereof limit the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristic or characteristics of the claimed invention.

As used herein, "shuttle" means a compound, polymer, complex, or mixture thereof that facilitates transport of phosphoinositides, inositol polyphosphates, and mixtures thereof into cells. Preferred shuttles comprise polyamines.

As used herein, "polyamines" includes aminoglycosidic aminocyclitols (e.g., aminoglycoside antibiotics), synthetic "spherical" dendrimeric polyamines, polybasic nuclear proteins (histones), polybasic polypeptides, lipidic polyamines, polyethyleneimine, steroidal polyamines, and the like, and mixtures thereof. As used herein, a "polybasic protein" or "polybasic polypeptide" contains sufficient lysine, arginine, and/or histidine residues to complex an anionic ligand, such as an IP_n or PIP_n. The polybasic polypeptide may also contain unnatural or non-protein amino acids, N-acylglycine groups, and any of a known group of amide group replacements known as peptide bond isosteres.

As used herein, "aminoglycosidic aminocyclitols" or "aminoglycosides" that can be used according to the present invention include, without limitation, neomycin, gentamicin, geneticin, streptomycin, kanamycin, tobramycin, spectinomycin, formicidin, streptamine, deoxystreptamine, epistreptamine, fortamine, validamine, valienamine, hydroxyvalidamine, valiciamine, validoxylamine A, validoxylamine B, validoxylamine G, and the like, and mixtures thereof. The primary or secondary amino groups of aminoglycosidic aminocyclitols can be reacted with isothiocyanato groups, with succinimidyl esters, with sulfonyl halides, and with other amine-reactive derivatives of fluorescent compounds according to the guidelines set out herein.

As used herein, suitable fluorescent compounds that can be used according to the present invention include chemically activated, tetherable analogs of acrylodan, AMCA, BODIPY, Cascade-Blue, CINERF, dansyl, dialkylaminocoumarin, eosin, erythrosine, fluorescein (FITC), hydroxycoumarin, NBD, Oregon green, PyMPO, pyrene, rhodamine, Rhodol Green, TMR, Texas Red, X-Rhodamine, and the like.

As used herein, "eukaryotic cells" and similar terms mean cells of animals and plants, as well as other cells containing membrane-bound nuclei and mitochondria.

As used herein, "prokaryotic cells" and similar terms mean cells of bacteria, blue-green algae, and other cells lacking nuclei and mitochondria.

As used herein, "bacteria" includes both Gram-negative and Gram-positive species. Exemplary of Gram-negative bacteria are species of the genera *Escherichia*, *Pseudomonas*, *Acinetobacter*, *Francisella*, *Bordetella*, *Shigella*, *Salmonella*, *Proteus*, *Yersinia*, *Klebsiella*,

Enterobacter, *Serratia*, *Vibrio*, *Haemophilus*, *Pasteurella*, *Streptobacillus*, *Bacteriodes*, *Fusobacterium*, *Neisseria*, and the like. Exemplary of Gram-positive bacteria are species of the genera *Staphylococcus*, *Streptococcus*, *Corynebacterium*, *Bacillus*, *Clostridium*, and the like. Also to be considered within the definition of bacteria are actinomycetes, such as the genera *Nocardia* and *Actinomyces*, mycobacteria (i.e., *Mycobacterium*), obligately intracellular bacteria such as rickettsiae and chlamydiae (e.g., *Rickettsia* and *Chlamydia*), spiral bacteria (e.g., *Spirillum*, *Campylobacter*, *Treponema*, *Borrelia*, *Leptospira*, and *Helicobacter*), and mycoplasmas (e.g., *Mycoplasma*).

As used herein, "parasite" includes protozoans, such as *Cryptosporidium* spp., *Toxoplasma* spp., *Trypanosoma* spp., *Giardia* spp., *Trichomonas* spp., *Entamoeba* spp., *Plasmodium* spp., *Balantidium coli*, *Pneumocystis* spp., *Leishmania* spp., *Acanthamoeba* spp., *Naegleria* spp., and the like; and helminths such as *Enterobius* spp., *Trichinella* spp., *Schistosoma* spp., *Ascaris* spp., *Trichuris* spp., *Ancyclostoma* spp., *Necator* spp., *Strongyloides* spp., *Trichostrongylus* spp., *Diphyllobothrium* spp., *Hymenolepsis* spp., *Wuchereria* spp., *Onchocerca* spp., *Loa* spp., *Taenia* spp., *Draculuncus* spp., *Paragonimus* spp., *Clonorchis* spp., *Fasciola* spp., *Fasciolopsis* spp., and the like.

As used herein, "fungi" includes *Microsporum* spp., *Trichophyton* spp., *Epidermophyton* spp., *Candida* spp., *Microsporum* spp., *Cryptococcus* spp., *Aspergillus* spp., *Malassezia* spp., *Coccidioides* spp., *Histoplasma* spp. *Mucor* spp., *Blastomyces* spp., *Sporotrichum* spp., *Torulopsis* spp., *Rhodotorula* spp., and the like.

As used herein, "substantially unaltered" means that uptake of an aminoglycoside antibiotic into a prokaryotic cell in the presence of a compound being tested for ability to reduce cytotoxicity to a mammalian cell is 90% or greater of the uptake of the aminoglycoside in the absence of the compound.

The chemical structures of the illustrative fluorescent phosphoinositide PtdIns(4,5)P_2-NBD and PtdIns(3,4,5)P_3-NBD, the illustrative fluorescent inositol polyphosphate Ins(1,4,5)P_3-XRITC, and two illustrative fluorescent polyamine shuttles, neomycin-XRITC (Neo-XR) and neomycin-Rhodamine B (Neo-RB), are shown in FIGS. 1a-d. The fluorescent PIP_ns are fully water-soluble at the concentrations employed. The attachment of the fluorophore to the acyl chain allows full recognition by target proteins of both the inositol polyphosphate head group and the glycerol backbone proximal to the headgroup. G.D. Prestwich et al., in *Phosphoinositides: Chemistry, Biochemistry and Biomedical Applications*

24-37 (K.S. Bruzik ed. 1999). All fluorescent and non-fluorescent shuttle and PIP_n derivatives have been synthetically prepared, G.D. Prestwich, Touching all the bases: Inositol polyphosphate and phosphoinositide affinity probes from glucose, 29 Acc. Chem. Res. 503-513 (1996), and are readily available (Echelon Research Laboratories, Salt Lake City, Utah), thus facilitating implementation of the present invention.

These polyamine-PIP_n complexes are preferably formed prior to addition to the extracellular medium of the cells. The molar ratio of PIP_n to polyamine is preferably in the range of about 1000:1 to 1:1000, more preferably 50:1 to 1:50, and most preferably 5:1 to 1:5. The localization of both the polyamine shuttle and the cargo PIP_n can be followed by monitoring one- or two-color fluorescent tags using laser scanning confocal microscopy. In a preliminary survey of several fluorescently-modified PIP_ns and IP_ns, the efficiency of fluorophore-PIP_n delivery was dependent on the polyamine shuttle, the number and location of phosphates in the PIP_n head group, the nature and length of the PIP_n acyl chains, and the cell type. For five mammalian cell lines examined (see Example 19), the most rapid and efficient cellular delivery of PtdIns(3,5)P_2, PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3 analogs was achieved using small aminoglycoside antibiotics such as neomycin and kanamycin, and was partly dependent on the number of amino groups available for complexation. Dendrimeric amines were nearly as efficient as neomycin, but caused cell detachment over several hours, perhaps due to disruption of intracellular PIP_n- cytoskeletal protein interactions. The histones were the least efficient shuttles, but did not cause any gross change in cell physiology; both cell division and redistribution of labeled PIP_ns was directly observable. Other polyamines examined were less efficient, but could still cause measurable but slow intracellular uptake in attached or detached cells.

In the absence of a polyamine shuttle, PIP_n analogs fail to enter healthy, attached cells to detectable levels within 30 minutes, as shown for NIH 3T3 cells in FIG. 2a (inset). In contrast, when a mixture of a PIP_n analog and a polyamine shuttle are added to cells, the PIP_n analog rapidly enters the cells. For animal cells, entry is typically within 30-60 seconds. FIG. 2a illustrates the histone-facilitated entry of PtdIns(4,5)P_2-NBD into NIH 3T3 fibroblast cells after 5 minutes. FIG. 2b shows histone-facilitated entry of Ins(1,4,5)P_3, and FIG. 2b (inset) is the control without histone. FIG. 2c shows histone-facilitated uptake of PtdIns(3,4,5)P_3, and the inset is at higher magnification.

These complexes could, in principle, enter by passive or active mechanisms. To test the hypothesis that the mechanism of cellular entry was passive movement across membranes due to charge neutralization, endocytosis was slowed (Chinese Hamster Ovary cells, or CHO cells) or blocked (Madin-Darby Canine Kidney cells, or MDCK cells) prior to addition of the shuttle-NBD-PIP_n complex or a membrane-specific fluorophore as control (see Example 23). Endocytosis was altered in cells by incubation at lowered pH (5.5-6.0), at low temperature (4°C) or in the presence of GTPγS. Under these conditions, the lipophilic styryl dye FM 4-64 remained in the plasma membrane of MDCK cells with no detectable intracellular accumulation. Upon release from the endocytic block by returning cells to standard growth medium, FM 4-64 was detectably endocytosed within 5-10 min. In CHO cells, a kinetic delay in endocytic vesicle movement was realized with the different treatments. Thus, for each of the three treatments, FM 4-64 movement into CHO cells was significantly delayed but not prevented, and, upon release from the block, FM 4-64 was rapidly internalized in the CHO cells. In contrast to the control experiments, blocking or slowing endocytosis in MDCK or CHO cells had no discernible effect on the kinetics of accumulation of the polyamine shuttle-PtdIns(4,5)P_2-NBD complex in these cell types. These data thus support a passive movement model across eukaryotic membranes.

Next, fluorescently-labeled shuttles were used in combination with PtdIns(4,5)P_2-NBD as the cargo to follow the separate subcellular destinations of the shuttle and the cargo in each of five cell lines and plant root tip cells (see Example 24). Intracellular localization of PtdIns(4,5)P_2-NBD and the fluorophore-conjugated aminoglycoside was confirmed by co-staining with organelle-specific fluorophores. A battery of organelle-specific fluorophores available commercially provided tools to examine organelles and membranes. As shown in FIGS. 3a and b, the fluorescent aminoglycoside derivative, Neo-RB, requires PtdIns(4,5)P_2 to achieve intracellular uptake. The fluor was concentrated in the endoplasmic reticulum (ER) and the nucleoli, consistent with its complexation to transfer RNA and ribosomal RNA subunits or their precursors. In other two-color images, the PIP_n analog, PtdIns(4,5)P_2-NBD, could be seen in the plasma membrane and in intracellular patterns consistent with its presence in the ER, Golgi, and nuclear membrane, and substructures within the nucleus (including the nucleoli). The co-localization of PtdIns(4,5)P_2-NBD and Neo-RB in nucleoli and in the ER is noteworthy. Indeed, these images suggest a method (described in more detail below) for screening for compounds that would minimize mammalian cytotoxicity of

aminoglycoside antibiotics and maximize bacterial toxicity, since for the first time it is possible to visually monitor both uptake and binding to high-affinity RNA targets in the ER and the nucleolus. This method would be complementary to the identification of RNA aptamers selected for binding to aminoglycosides. G. Werstuck & M. Green, Controlling gene expression in living cells through small molecule-RNA interactions, 282 Science 296-298 (1998).

To obtain data on the general applicability of this methodology for eukaryotic cells, several combinations of PIP_n-NBD analogs and shuttles were applied to plant cells. A dramatic delivery of PtdIns(4,5)P_2-NBD to the plasma membrane of living plant cells was observed (photograph not shown). The widely utilized model plant, *Arabidopsis thaliana,* D.W. Meinke et al., *Arabidopsis thaliana:* A model plant for genome analysis, 282 Science 662-667 (1998), was grown in liquid culture and fluorescent probes were delivered to roots of intact plantlets. To illustrate the general structure of the A. *thaliana* root tip cells, a cytoplasmic stain (Syto 11) and membrane stain (FM 4-64) were utilized. Since plant cells endocytose membrane slowly or not at all, the plasma membrane staining with FM4-64 was nearly static. When a mixture of PtdIns(4,5)P_2-NBD and Neo-RB was delivered to these cells, both fluorophores were immediately apparent in separate compartments of the root cells. The Neo-RB clearly stained the cell wall and to a lesser degree the plasma membrane, while the PtdIns(4,5)P_2-NBD moved on into cell plasma membranes. Importantly, omission of the shuttle resulted in very slow and inefficient uptake of the PtdIns(4,5)P_2-NBD. Finally, delivery of the PtdIns(4,5)P_2-NBD compound to the budding yeast, *Saccharomyces cerevisiae,* using a neomycin shuttle, has also been obtained, but uptake was slower than in animal and plant cells. Staining of the plant and fungal cells walls by NeoRB or other fluorescently labeled aminoglycosides, in the absence or presence of PIP_ns, allowed real time monitoring of endocytic vesicles in a hitherto unprecedented way, an important observation for basic research studies.

Important to the study of pathogens is the ability to deliver fluorescent aminoglycosides and phosphoinositides selectively to prokaryotic and parasite cells. FIG. 4a shows that fluorescent neomycin (FITC-labeled) was delivered into *E. coli* cells as a complex with PtdIns(4,5)P_2. FIG. 4b shows PtdIns(4,5)P_2-NBD was delivered into *E. coli* cells as a complex with histone protein. In FIG. 4c, a neomycin-rhodamine/PtdIns(4,5)P_2 complex entered the protozoan pathogen, *Cryptosporidium parvum* (bright spots among the field of

epithelial cells). In FIG. 4d, a PtdIns(4,5)P_2-NBD/histone complex rapidly entered *C. parvum*. These micrographs were obtained with a laser scanning confocal microscope 5 minutes after addition of the neomycin-FITC/PtdIns(4,5)P_2, PtdIns(4,5)P_2-NBD/histone, or neomycin-rhodamine/PtdIns(4,5)P_2 complexes to the cell media. The *C. parvum* cells were cultured on cover slips coated with bovine epithelial cells. In all of FIGS. 4a-d, the bright fluorescence is from the fluorophores (FITC, NBD, and Rhodamine) attached to neomycin (FIGS. 4 a and c) or PtdIns(4,5)P_2 (FIGS. 4 b and d).

To determine what happens to intracellular PtdIns(4,5)P_n-NBD when the cell physiology is altered, cells were loaded with PtdIns(4,5)P_n-NBD according to the present invention and then subjected to several treatments known to stimulate intracellular signaling pathways involving phosphoinositides. CHO, MDCK, NIH 3T3, and 3T3-L1 cells were treated with the relatively non-specific phospholipase C activating peptides, mastoparan and bradykinin. Because bradykinin works differentially on cell types containing distinct seven membrane pass (G-protein coupled) receptors (B1, B2, B3), the results were variable, with some cells types responding to 1 μM bradykinin with a modest relocalization of intracellular PtdIns(4,5)P_2-NBD and significant loss of fluorescent signal within minutes. Other cell types showed little or no response to bradykinin. The results obtained with mastoparan were similar to those obtained with bradykinin. Studies with human neutrophils also establish changes in intracellular PIP_ns introduced by the shuttle system occur in predicted fashion upon stimulation with growth factors and peptides such as formyl-Met-Leu-Phe.

A highly informative PtdIns(4,5)P_2-NBD redistribution experiment was conducted in which insulin was employed to activate cells. First, 3T3-L1 adipocytes (serum starved) were pre-loaded with an PtdIns(4,5)P_2-NBD/C6-histone complex. PtdIns(4,5)P_2-NBD was chosen because activation of receptor tyrosine kinases and PI 3-kinase could lead to conversion of PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 by PI 3-kinase, or PtdIns(4,5)P_2 could act as a substrate for phospholipase C isozymes (δ or γ). Both enzymatic activities are known to be modulated by PIP_n binding in these cells. The histone protein was chosen as the polyamine shuttle because high concentrations of aminoglycoside antibiotics are known to perturb cellular phospholipase activity. Thus, a complex of PtdIns(4,5)P_2-NBD (10 μM) and histone (3 μM) was delivered to cells and intracellular localization was monitored until equilibrium was reached. Ten minutes after loading of the shuttle-cargo complex, cells were treated with 60 nM insulin and confocal images were obtained at 10-sec intervals for 20 min. For the

duration of the time course of image collection, the same optical section was maintained. At 0 sec, the equilibrium localization of PtdIns(4,5)P_2-NBD to the plasma membrane, nucleus, and punctate and filamentous structures in the cytosol was clearly visible. After 2 min, modest decreases in the plasma membrane staining and relocalization of the fluorophore in the cytosol were observed. Six minutes after addition of insulin to the cells, nuclear and plasma membrane staining was dramatically decreased, and redistribution of the fluorophore in the cytosol reached a maximum. In the cytosol, the NBD fluorophore relocalized in a polar perinuclear pattern that was consistent with ER/Golgi staining.

The dramatic redistribution of the NBD fluorophore is intriguing, and two interpretations are possible. First, it is possible that the PI 3-kinase product, PtdIns(3,4,5)P_3-NBD, moves from the plasma membrane to intracellular sites. However, the second hypothesis is favored, in which the diffuse perinuclear-localized fluorophore would be diacylglyceryl-NBD, which is derived from phospholipase C action on PtdIns(4,5)P_2-NBD. E. Tall et al., Phosphoinositide binding specificity among phospholipase C isozymes as determined by photo-cross-linking to novel substrate and product analogs, 36 Biochemistry 7239-7248 (1997); M. Falasca et al., Activation of phospholipase C gamma by PI 3-kinase-induced PH domain-mediated membrane targeting, 17 EMBO J. 414-422 (1998). Recently published evidence supports a role for PIP_ns in the recruitment of phospholipase C isoforms (δ and γ) to the plasma membrane where a readily available pool of substrate exists. E. Tall et al., *supra*; Y.S. Bae et al., Activation of phospholipase C-gamma by phosphatidylinositol 3,4,5-trisphosphate, 273 J. Biol. Chem. 4465-4469 (1998).

The presently claimed method can also be used to introduce Ins(1,4,5)P_3 into cells for observing calcium flux. Cover slip-attached 3T3-L1 preadipocytes were loaded with the calcium indicator, calcium crimson, using a 15-minute incubation period. This dye exhibits a red fluorescence that increases in intensity upon calcium binding. Medium was exchanged and cells were observed until no fluorescence changes were evident. Then, a complex of Ins(1,4,5)P_3 and histone was added to the medium. A time-course collection of images showed an increase in red fluorescence within 10 seconds in the cytosol and nuclei of the treated cells. After 10 minutes, the red fluorescence of the calcium-calcium crimson complex was lower than when Ins(1,4,5)P_3 was first added. These data illustrate the utility of the present shuttle method for intracellular delivery of soluble inositol phosphates, and thus for

monitoring cellular events, such as calcium mobilization, which occur in response to increases in intracellular inositol phosphate concentrations.

In summary, the use of the shuttle-cargo system to ferry affinity-tagged or native phosphoinositides into living cells provides a simple method to examine the action, localization, and metabolism of phosphoinositides involved in signal transduction pathways in the context of changes in cell physiology. The availability of cell-permeant PIP_ns allows direct observation in time and space of specific phosphoinositide signaling events occurring in eukaryotic cells during growth and differentiation, following oncogenic transformation, and during pathogen infection. In addition, an important corollary is that PIP_ns can be used to shuttle aminoglycosides and other biologically active polyamines specifically into eukaryotic cells. Given the current critical need for new antibiotic strategies, this new technique should stimulate the pursuit of molecules that could reclaim the use of many aminoglycosides for which toxicity or resistance has reduced their clinical utility.

It will be realized by one skilled in the art that these methods provide the basis for implementing high throughput screening (HTS) for identifying agonists and antagonists for protein kinases and phosphoinositide kinases and for phosphoinositide and inositol phosphate binding proteins that are regulated by PIP_ns or IP_ns and may serve as downstream effectors in signaling pathways important for therapeutic interventions. These assays include cell-based assays using intracellular PIP_ns introduced by the shuttling system and could use primary cells, immortalized cells, cancer cells, cells transformed with plasmids encoding key enzymes or other proteins, and the like. The assays could also use *in vitro* cell extracts or partially purified or homogeneous proteins.

Examples

Fluorescent derivatives of polyamines were synthesized from the amine and either the isothiocyanate or succinimidyl ester derivatives of the fluorescent reagent in 1 N TEAB (aqueous triethylammonibicarbonate, pH 7.5). All compounds were purified by HPLC and their structures were confirmed by NMR and Electron Spray Mass Spectrometry (ES-MS).

Example 1

This example describes the synthesis of the fluorophore-conjugated polyamine, neomycin-rhodamine B (Neo-RB; FIG. 1d). J.B.-H. Tok & R.R. Rando, Simple aminols as

aminoglycoside surrogates, 120 J. Amer. Chem. Soc. 8279-8280 (1998), describes preparation of certain fluorescent aminoglycosides and their use in screening to find RNA-interactive simple aminols.

Neomycin trisulfate (110 mg, 0.121 mmol) and rhodamine B isothiocyanate (8.2 mg, 0.015 mmol; Aldrich Chemical Co., St. Louis, Missouri) were dissolved in 2 mL of 1.0 M TEAB (triethylammonium bicarbonate, pH 8.6) and 0.2 mL of dimethylformamide (DMF). The solution was stirred for 72 hr at 25°C. The solution was then concentrated *in vacuo*, the residue was dissolved in water (10 mL), and byproducts were removed with two 10-mL methylene chloride extractions. The aqueous solution was again concentrated *in vacuo*, and reversed-phase (C_{18}) HPLC fractions containing purified Neo-RB were collected using an acetonitrile gradient in 0.06% trifluoroacetic acid, and were lyophilized. ES-MS revealed an M^{+2} peak for the tetraprotonated species: *mz* 511.4 (calculated for M+4H, 1023.3).

Example 2

In this example, Neo-XR (FIG. 1c) was prepared according to the procedure of Example 1 except that X-RITC (Molecular Probes, Inc., Eugene, Oregon) was used as the activated fluorescent reagent instead of Rhodamine B isothiocyanate.

Example 3

In this example, a dendrimer amine with 12 primary amines was fluorescently labeled according to the procedure of Example 1 except that the dendrimer amine was substituted for neomycin. The dendrimer amine was prepared by the reduction of 12-cascade:amino(3):(1-azapropylidene):(1-azabutylidene):2-propanenitrile, R. Moors & F.Vogtle, Dendrimere polyamine, 126 Chem. Ber. 2133-2135 (1993) (hereby incorporated by reference), with di-isobutylaluminum hydride in methylene chloride.

Example 4

In this example, the procedure of Example 1 was followed except that the dendrimer amine, DAB-Am-32 (Aldrich Chemical Co., St. Louis, Missouri), was substituted for neomycin.

Example 5

In this example, the procedure of Example 1 was followed except that histone Type III-S from calf thymus (Sigma Chemical Co., St. Louis, Missouri) was substituted for neomycin.

Example 6

In this example, the procedure of Example 1 was followed except that fluorescein isothiocyanate (FITC) was substituted for Rhodamine B isothiocyanate and the solution was stirred for 1 hr and kept standing for 36 hr at room temperature. ES-MS, *m/z* 1003.63 Calcd 1004.2.

Example 7

In this example, the procedure of Example 6 was followed except that SNAFL-NHS was substituted for FITC and the solution was stirred for 12 hr and kept standing for 7 days. ES-MS, *m/z* 1002.19 Calcd 1022.

Example 8

In this example, the procedure of Example 6 was followed except that geneticin disulfate was substituted for neomycin, and the solution was stirred for 1 day. ES-MS, *m/z* 887.21 Calcd 885.9.

Example 9

In this example, the procedure of Example 6 was followed except that gentamicin (mixture of gentamicin C_1, C_2, C_{1a}) monosulfate was substituted for neomycin. ES-MS, *m/z* 868, 853, 838 Calcd 866.55 (gentamicin C_1), 853 (gentamicin C_2), 838 (gentamicin C_{1a}).

Example 10

In this example, the procedure of Example 6 was followed except that kanamycin was substituted for neomycin. The results were substantially similar to those obtained with neomycin.

Example 11

In this example, the procedure of Example 6 was followed except that paromomycin was substituted for neomycin. The results were substantially similar to those obtained with neomycin.

Example 12

In this example, the procedure of Example 6 was followed except that streptomycin was substituted for neomycin. The results were substantially similar to those obtained with neomycin.

Example 13

In this example, the procedure of Example 6 was followed except that tobramycin was substituted for neomycin. The results were substantially similar to those obtained with neomycin.

Example 14

Synthesis of fluorescent PtdInsP$_n$ derivatives. *sn*-2-*O*-Stearoyl PtdIns(4,5)P_2-NBD (C_{18})was synthesized as previously described, J. Chen et al., Synthesis of photoactivatable 1,2-*O*-diacyl-*sn*-glycerol derivatives of 1-L-phosphatidyl-D-myo-inositol 4,5-bisphosphate and 3,4,5-trisphosphate, 61 J. Org. Chem. 6305-6312 (1996) (hereby incorporated by reference). NBD-X-SE was purchased from Molecular Probes, Inc. (Eugene, Oregon).

Example 15

In this example, the procedure of Example 14 was followed except a shorter chain *sn*-1-*O*-(6-aminohexanoyl) *sn*-2-*O*-hexanoyl derivative was prepared. Reaction with NBD-X-SE was performed as described to produce the PtdIns(4,5)P_2-NBD (C_6), which was characterized by proton NMR.

Example 16

In this example, the procedure of Example 14 was followed except that an aminoacyl derivative of PtdIns(3,4,5)P_3 was substituted for the aminoacyl derivative of PtdIns(4,5)P_2.

Example 17

In this example, the procedure of Example 14 was followed except that an aminoacyl derivative of PtdIns(3,5)P_2 was substituted for an aminoacyl derivative of PtdIns(4,5)P_2, and BODIPY-FL or BODIPY-5G1 was substituted for NBD.

Example 18

In this example, the procedure of Example 14 was followed except that Texas Red was substituted for NBD.

Example 19

Delivery of analogs to cells. COS-7, Madin-Darby canine kidney (MDCK), NIH 3T3 mouse fibroblasts, and 3T3-L1 adipocyte cells were cultured (DMEM 90%, calf serum 10%) on 10- mm diameter cover slips for 12-24 hr prior to phosphoinositide delivery experiments. Chinese hamster ovary (CHO) cells were cultured on cover slips in Ham's F12 medium with 5% calf serum. For the phosphoinositide delivery experiments, dyes were added to the medium (approximately 20 μl) on cover slips that were mounted directly to slides or to glass plates with a 200-μl capacity well. Immediately following addition of the dyes, the glass plate or slide was mounted on the microscope stage for observation.

Cells were examined using an inverted microscope (Nikon) and a BioRad laser scanning confocal microscope system (MRC 1024) with Laser Sharp acquisition software. The 488 nm laser line was used for excitation of NBD-conjugated PIP_ns. The 568 nm laser line was used for Neo-XR and Neo-RB. Images of animal cells were collected using a 60X oil immersion objective. No post-acquisition enhancement of images was performed, except for gray-scale conversion to color using Laser Sharp software or Confocal Assistant software.

When no polyamine shuttle was added to the PtdIns(4,5)P_2-NBD, individual cells appeared as dark ghosts or multicellular clusters (FIG. 2a (inset)), indicating that the PIP_n analog failed to enter the cells to detectable levels within 30 minutes. When PtdIns(4,5)P_2-NBD was mixed with a solution of histone protein prior to being added to the cells, fluorescence in the cells was easily detected within 5 minutes, illustrating histone-facilitated entry of the PIP_n analog into the cells (FIG. 2a). The fluorescence was generally cytoplasmic with some nuclear, nuclear envelope, and nucleosomal accumulation. Similar results were obtained using histone as a carrier for PtdIns(3,4,5)P_3 and Ins(1,4,5)P_3-XRITC.

Example 20

In this example, the procedure of Example 19 was followed except that Ins(1,4,5)P_3 was substituted for PtdIns(4,5)P_2-NBD.

Example 21

In this example, the procedure of Example 19 was followed except that XRITC-Ins(1,4,5)P_3 was substituted for PtdIns(4,5)P_2-NBD.

Example 22

In this example, the procedure of Example 19 was followed except that PtdIns(3,4,5)P_3 was substituted for PtdIns(4,5)P_2-NBD.

Example 23

To determine whether movement of PIP_n-polyamine shuttle into cells was due to endocytosis or crossing the membrane passively, endocytosis was slowed or blocked. CHO and MDCK cells were incubated in low pH medium (5.5-6.0), at low temperature (4°C), or in the presence of GTPγS to alter endocytosis. Localization of the lipophilic styryl dye, FM 4-64, in the plasma membrane and its movement into cells was monitored via time course optical section collection by confocal microscopy. When cells were released from one of the endocytic blocks by returning them to standard growth medium, FM 4-64 was detectably endocytosed within 5-10 min.

During simultaneous delivery experiments, FM 4-64 movement was monitored concomitantly with phosphoinositide analog/shuttle solutions. In MDCK cells, endocytosis was blocked and the phosphoinositide analog/shuttle complexes still rapidly entered cells. In CHO cells, endocytosis was slowed and phosphoinositide analog/shuttle complexes still rapidly entered cells.

Thus, under these conditions, the lipophilic styryl dye FM 4-64 remained in the plasma membrane of MDCK cells with no detectable intracellular accumulation. Upon release from the endocytic block by returning cells to standard growth medium, FM 4-64 was detectably endocytosed within 5-10 min. In CHO cells, a kinetic delay in endocytic vesicle movement was realized with the different treatments. Thus, for each of the three treatments, FM 4-64 movement into CHO cells was significantly delayed but not prevented; upon release

from the block, FM 4-64 rapidly internalized in the CHO cells. In contrast to the control experiments, blocking or slowing endocytosis in MDCK or CHO cells had no discernible effect on the kinetics of accumulation of the polyamine shuttle- PtdIns(4,5)P_2-NB D complex in these cell types. These data thus support a passive movement model across eukaryotic membranes.

Example 24

Organelle-specific stains and endocytosis. Intracellular localization of the phosphoinositide-conjugated fluorophores and aminoglycoside-conjugated fluorophores was determined in five animal cell lines and plant root tip cells by co-localization with commercially available organelle-specific fluorescent dyes. Staining of organelles was performed simultaneously or in side-by-side experiments using organelle-specific fluorophores. All organelle stains were obtained from Molecular Probes. In this study, $DiOC_6$ (ER), BODIPY TR ceramide (Golgi), Acridine orange (nucleic acids), DAPI (DNA), and Syto11 (nucleic acids) were used to stain intracellular organelles. FM 4-64 was used to stain the plasma membrane, endosomes, and lysosomes.

Separate destinations were observed for the shuttles and phosphoinositide cargo molecules in two-color images. A 1: 1 or 1:2 molar equivalent mixture of PtdIns(4,5)P_2-NBD and Neo-RB was added to the cell media immediately prior to recording confocal images of the cells attached to cover slips. The uptake of the 1: 1 complex of PtdIns(4,5)P_2-NBD with Neo-RB was documented for CHO cells and of the 1: 1 complex of PtdIns(4,5)P_2-NBD with Neo-XR into 3T3-L1 adipocytes. The location of the fluorescent dyes appears to reach equilibrium within 5 min incubation for most cells and complexes tested. The red Neo-RB appears concentrated in the endoplasmic reticulum (ER) and the nucleoli, consistent with its complexation to transfer RNA and ribosomal RNA subunits. The green PtdIns(4,5)P_2-NBD can be seen in the plasma membrane and in intracellular patterns consistent with its presence in the ER, Golgi, the nuclear membrane, and substructures within the nucleus (including the nucleoli).

The dramatic co-localization (yellow) of the PtdIns(4,5)P_2-NBD and Neo-RB in nucleoli and in the ER is particularly noteworthy. This suggests that the integrity of the complex is largely retained during the minutes required for the complex to transit the cell

membrane, move through the cytosol, and then either concentrate in the ER or to traverse the nuclear membrane and bind stably to the nucleoli.

Example 25

Whole *Arabidopsis thaliana* plants were grown in liquid medium (0.5X Murashige and Skoog, IX B5 vitamins, 2% glucose). For imaging experiments, intact one- to two-week-old whole plants were placed in the glass plate wells (total volume 200 *μl)* and fluorescent shuttle-cargo complexes were added. Plants were kept submerged and intact for all experiments, by covering the top of the well with a cover slip after fluorophore addition. Images of plant cells were collected 10-15 min following addition of the fluorescent PIP_n-fluorescent neomycin complex. Microscopy was carried out according to the procedure described above; a 40X objective was used for some of the plant cell images. The results of this experiment showed that PtdIns(4,5)P_2-NBD was delivered to the plasma membrane of the plant cells in less than 10 minutes and did not move into other intracellular membranes during a 60-minute observation period.

Example 26

In this example, the procedure of Example 24 was carried out, except the cells used were the yeast, *Saccharomyces cerevisiae*. Uptake was achieved, but the rate of uptake was slower than with animal or plant cells. These results show that the shuttle facilitated entry of PtdIns(4,5)P_2-NBD into fungal cells.

Example 27

In this example, the procedure of Example 24 was carried out, except the cells used were *E. coli* cells and the shuttle was histone. The results obtained from this experiment showed that the carrier polyamine facilitated entry of PtdIns(4,5)P_2-NBD into bacterial cells (FIGS. 4a and 4b), and *vice versa*, that PtdIns(4,5)P_2 facilitated uptake of polyamines, such as histone or fluorescently labeled aminoglycosides, into bacterial cells.

Example 28

In this example, the procedure of Example 24 was carried out, except the cells used were *Cryptosporidium parvum*. The results obtained from this experiment showed that the

shuttle facilitated entry of the PtdIns(4,5)P_2-NBD into protozoal cells in preference to human epithelial cells (FIGS. 4c and 4d). Moreover, the labeled aminoglycoside was selectively delivered to pathogen cells in comparison to human host cells.

CLAIMS

We claim:

1. A composition of matter comprising a mixture of (a) a phosphoinositide polyphosphate, inositol polyphosphate, or mixture thereof, and (b) a polyamine.

2. The composition of claim 1 wherein said polyamine is a member selected from the group consisting of aminoglycosides, dendrimeric polyamines, histones, polybasic polypeptides, lipidic polyamines, polyethyleneimine, steroidal polyamines, and mixtures thereof.

3. The composition of claim 2 wherein at least one of said phosphoinositide polyphosphate, inositol polyphosphate, or mixture thereof and polyamine is labeled with a detectable label.

4. The composition of claim 3 wherein said label is a member of the group consisting of fluorescent labels, radiolabels, chemiluminescent labels, spin labels, photophores, chromophores, nanogold particles, biotin, and mixtures thereof.

5. The composition of claim 4 wherein said label is a fluorescent label.

6. The composition of claim 5 wherein said fluorescent label is a member selected from the group consisting of acrylodan, AMCA, BODIPY, Cascade-Blue, CINERF, dansyl, dialkylaminocoumarin, eosin, erythrosine, fluorescein, hydroxycoumarin, NBD, Oregon green, PyMPO, pyrene, rhodamine, Rhodol Green, TMR, Texas Red, and X-Rhodamine.

7. The composition of claim 6 wherein said fluorescent label is NBD.

8. The composition of claim 6 wherein said fluorescent label is BODIPY.

9. A method for facilitating uptake of a phosphoinositide polyphosphate, inositol polyphosphate, or mixture thereof into a cell comprising contacting said cell with a composition of matter comprising a mixture of (a) a phosphoinositide polyphosphate, inositol polyphosphate, or mixture thereof, and (b) a polyamine.

10. The method of claim 9 wherein said cell is a eukaryotic cell.

11. The method of claim 10 wherein said eukaryotic cell is an animal cell.

12. The method of claim 10 wherein said eukaryotic cell is a plant cell.

13. The method of claim 10 wherein said eukaryotic cell is a member selected from the group consisting of protozoal cells, helminthic cells, and fungal cells.

14. The method of claim 9 wherein said cell is a prokaryotic cell.

15. The method of claim 9 wherein said polyamine is a member selected from the group consisting of aminoglycosides, dendrimeric polyamines, histones, polybasic polypeptides, lipidic polyamines, polyethyleneimine, steroidal polyamines, and mixtures thereof.

16. The method of claim 15 wherein at least one of said phosphoinositide polyphosphate, inositol polyphosphate, or mixture thereof and polyamine is labeled with a detectable label.

17. The method of claim 16 wherein said label is a member of the group consisting of fluorescent labels, radiolabels, chemiluminescent labels, spin labels, photophores, chromophores, nanogold particles, biotin, and mixtures thereof.

18. The method of claim 17 wherein said label is a fluorescent label.

19. The method of claim 18 wherein said fluorescent label is a member selected from the group consisting of acrylodan, AMCA, BODIPY, Cascade-Blue, CINERF, dansyl, dialkylaminocoumarin, eosin, erythrosine, fluorescein, hydroxycoumarin, NBD, Oregon green, PyMPO, pyrene, rhodamine, Rhodol Green, TMR, Texas Red, and X-Rhodamine.

20. The method of claim 19 wherein said fluorescent label is NBD.

21. The method of claim 19 wherein said fluorescent label is BODIPY.

22. A method for facilitating uptake of an aminoglycoside antibiotic into a cell comprising contacting said cell with a composition of matter comprising a mixture of (a) a phosphoinositide polyphosphate, inositol polyphosphate, or mixture thereof, and (b) said aminoglycoside antibiotic.

23. The method of claim 22 wherein said cell is a eukaryotic cell.

24. The method of claim 23 wherein said eukaryotic cell is an animal cell.

25. The method of claim 23 wherein said eukaryotic cell is a plant cell.

26. The method of claim 23 wherein said eukaryotic cell is a member selected from the group consisting of protozoal cells, helminthic cells, and fungal cells.

27. The method of claim 22 wherein said cell is a prokaryotic cell.

28. The method of claim 27 wherein said prokaryotic cell is a member of the group consisting of the genera *Escherichia*, *Pseudomonas*, *Acinetobacter*, *Francisella*, *Bordetella*, *Shigella*, *Salmonella*, *Proteus*, *Yersinia*, *Klebsiella*, *Enterobacter*, *Serratia*, *Vibrio*, *Haemophilus*, *Pasteurella*, *Streptobacillus*, *Bacteriodes*, *Fusobacterium*, *Neisseria*, *Staphylococcus*, *Streptococcus*, *Corynebacterium*, *Bacillus*, *Clostridium*, *Nocardia*,

Actinomyces, *Mycobacterium*, *Rickettsia*, *Chlamydia*, *Spirillum*, *Campylobacter*, *Treponema*, *Borrelia*, *Leptospira*, *Helicobacter*, and *Mycoplasma*.

29. The method of claim 22 wherein said aminoglycoside antibiotic is a member selected from the group consisting of neomycin, gentamicin, geneticin, streptomycin, kanamycin, tobramycin, spectinomycin, formicidin, streptamine, deoxystreptamine, epistreptamine, fortamine, validamine, valienamine, hydroxyvalidamine, valiciamine, validoxylamine A, validoxylamine B, validoxylamine G, and mixtures thereof.

30. A method for visualizing the uptake and localization of an aminoglycoside in a cell comprising:

(a) mixing an aminoglycoside bearing a visually identifiable label with a polyamine to result in a mixture;

(b) contacting the cell with the mixture such that the mixture is delivered into the cell; and

(c) observing the label, thereby visualizing the uptake and localization of the aminoglycoside in the cell.

31. The method of claim 30 wherein said cell is a eukaryotic cell.

32. The method of claim 31 wherein said eukaryotic cell is an animal cell.

33. The method of claim 31 wherein said eukaryotic cell is a plant cell.

34. The method of claim 31 wherein said eukaryotic cell is a member selected from the group consisting of protozoal cells, helminthic cells, and fungal cells.

35. The method of claim 30 wherein said cell is a prokaryotic cell.

36. The method of claim 35 wherein said prokaryotic cell is a member of the group consisting of the genera *Escherichia*, *Pseudomonas*, *Acinetobacter*, *Francisella*, *Bordetella*, *Shigella*, *Salmonella*, *Proteus*, *Yersinia*, *Klebsiella*, *Enterobacter*, *Serratia*, *Vibrio*, *Haemophilus*, *Pasteurella*, *Streptobacillus*, *Bacteriodes*, *Fusobacterium*, *Neisseria*, *Staphylococcus*, *Streptococcus*, *Corynebacterium*, *Bacillus*, *Clostridium*, *Nocardia*, *Actinomyces*, *Mycobacterium*, *Rickettsia*, *Chlamydia*, *Spirillum*, *Campylobacter*, *Treponema*, *Borrelia*, *Leptospira*, *Helicobacter*, and *Mycoplasma*.

37. The method of claim 30 wherein said aminoglycoside antibiotic is a member selected from the group consisting of neomycin, gentamicin, geneticin, streptomycin, kanamycin, tobramycin, spectinomycin, formicidin, streptamine, deoxystreptamine, epistreptamine, fortamine, validamine, valienamine, hydroxyvalidamine, valiciamine,

validoxylamine A, validoxylamine B, validoxylamine G, and mixtures thereof.

38. The method of claim 30 wherein said label is a fluorescent label.

39. The method of claim 38 wherein said fluorescent label is a member of the group consisting of acrylodan, AMCA, BODIPY, Cascade-Blue, CINERF, dansyl, dialkylaminocoumarin, eosin, erythrosine, fluorescein, hydroxycoumarin, NBD, Oregon green, PyMPO, pyrene, rhodamine, Rhodol Green, TMR, Texas Red, and X-Rhodamine.

40. The method of claim 39 wherein said fluorescent label is NBD.

41. The method of claim 39 wherein said fluorescent label is BODIPY.

42. A method for screening for a compound that minimizes cytotoxicity of aminoglycoside antibiotics to mammalian cells comprising:

(a) mixing an aminoglycoside bearing a label with a polyamine to result in a mixture;

(b) contacting a mammalian cell with the mixture and with a compound to be tested such that, absent the presence of the compound, the mixture would be delivered into the cell; and

(c) observing the label, thereby observing the uptake and localization of the aminoglycoside in the mammalian cell, wherein reduced uptake of the aminoglycoside or altered localization of the aminoglycoside as compared to the cell treated with the mixture in the absence of the compound indicates reduced cytotoxicity.

43. The method of claim 42 wherein said aminoglycoside antibiotic is a member selected from the group consisting of neomycin, gentamicin, geneticin, streptomycin, kanamycin, tobramycin, spectinomycin, formicidin, streptamine, deoxystreptamine, epistreptamine, fortamine, validamine, valienamine, hydroxyvalidamine, valiciamine, validoxylamine A, validoxylamine B, validoxylamine G, and mixtures thereof.

44. The method of claim 42 wherein said label is a member of the group consisting of fluorescent labels, radiolabels, chemiluminescent labels, spin labels, photophores, chromophores, nanogold particles, biotin, and mixtures thereof.

45. The method of claim 44 wherein said label is a fluorescent label.

46. The method of claim 45 wherein said fluorescent label is a member of the group consisting of acrylodan, AMCA, BODIPY, Cascade-Blue, CINERF, dansyl, dialkylaminocoumarin, eosin, erythrosine, fluorescein, hydroxycoumarin, NBD, Oregon green, PyMPO, pyrene, rhodamine, Rhodol Green, TMR, Texas Red, and X-Rhodamine.

47. The method of claim 46 wherein said fluorescent label is NBD.

48. The method of claim 46 wherein said fluorescent label is BODIPY.

49. The method of claim 42 further comprising determining whether uptake of the aminoglycoside into a bacterial cell is substantially unaltered in the presence of the compound.

50. A composition of matter comprising an aminoglycoside antibiotic covalently bonded to a fluorescent compound.

51. The composition of claim 50 wherein said aminoglycoside antibiotic is a member selected from the group consisting of neomycin, gentamicin, geneticin, streptomycin, kanamycin, tobramycin, spectinomycin, formicidin, streptamine, deoxystreptamine, epistreptamine, fortamine, validamine, valienamine, hydroxyvalidamine, valiciamine, validoxylamine A, validoxylamine B, validoxylamine G, and mixtures thereof.

52. The composition of claim 50 wherein said fluorescent compound is a member selected from the group consisting of acrylodan, AMCA, BODIPY, Cascade-Blue, CINERF, dansyl, dialkylaminocoumarin, eosin, erythrosine, fluorescein, hydroxycoumarin, NBD, Oregon green, PyMPO, pyrene, rhodamine, Rhodol Green, TMR, Texas Red, and X-Rhodamine.

53. The composition of claim 50 wherein said aminoglycoside antibiotic is covalently bonded to said fluorescent compound through a linker moiety.

54. The composition of claim 53 wherein said linker moiety is an isothiocyanate or succinimidyl ester group.

55. The composition of claim 50 wherein said aminoglycoside antibiotic is neomycin and said fluorescent compound is rhodamine B.

56. The composition of claim 50 wherein said aminoglycoside antibiotic is neomycin and said fluorescent compound is XR.

57. A method for monitoring calcium flux in a cell comprising:

(a) loading the cell with a calcium indicator, exchanging the medium, and monitoring the cell until no intensity change in the calcium indicator is observed;

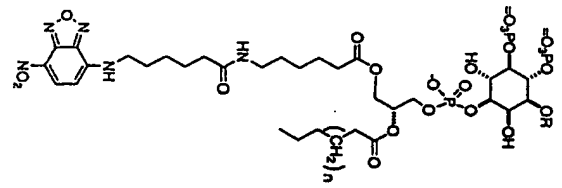
(b) then contacting the cell with a complex of Ins(1,4,5)P_3 and a shuttle such that the Ins(1,4,5)P_3 enters the cell and thereby modulates the calcium flux thereof; and

(c) detecting the calcium indicator wherein a change in the intensity of the calcium indicator indicates a change in calcium flux in the cell.

58. The method of claim 57 wherein the calcium indicator is a member selected from the group consisting of calcium crimson and Fluo-3 and the change in intensity is detected as a change in fluorescence.

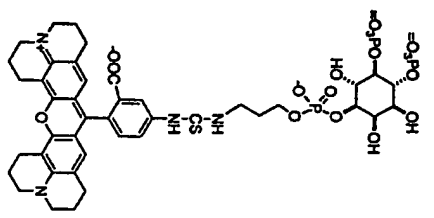
59. The method of claim 57 wherein the shuttle is a polyamine selected from the group consisting of aminoglycosides, dendrimeric polyamines, histones, polybasic polypeptides, lipidic polyamines, polyethyleneimine, steroidal polyamines, and mixtures thereof.

60. The method of claim 59 wherein said polyamine is a histone.



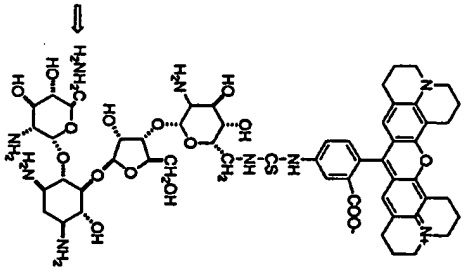
R =H, PtdIns(4,5)P_2-NBD C_8 (n = 1) and C_{18} (n = 13)
R =PO_3=,PtdIns(3,4,5)P_3-NBD C_{16} (n =11)

FIG. 1a



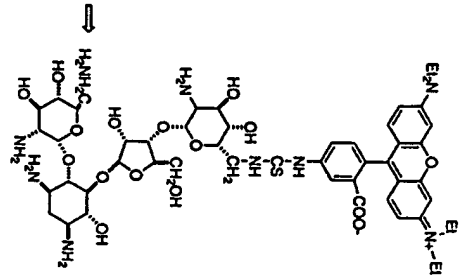
Ins(1,4,5)P_3-XRITC

FIG. 1b



Neomycin-XRITC (Neo-XR)

FIG. 1c



Neomycin-Rhodamine B (Neo-RB)

FIG. 1d

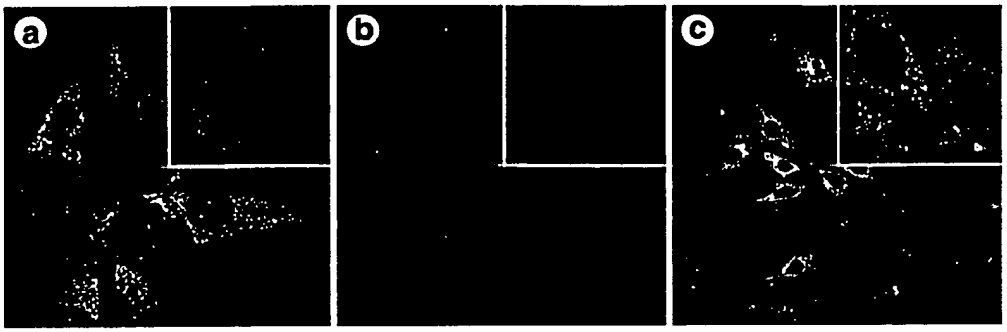


FIG. 2a FIG. 2b FIG. 2c

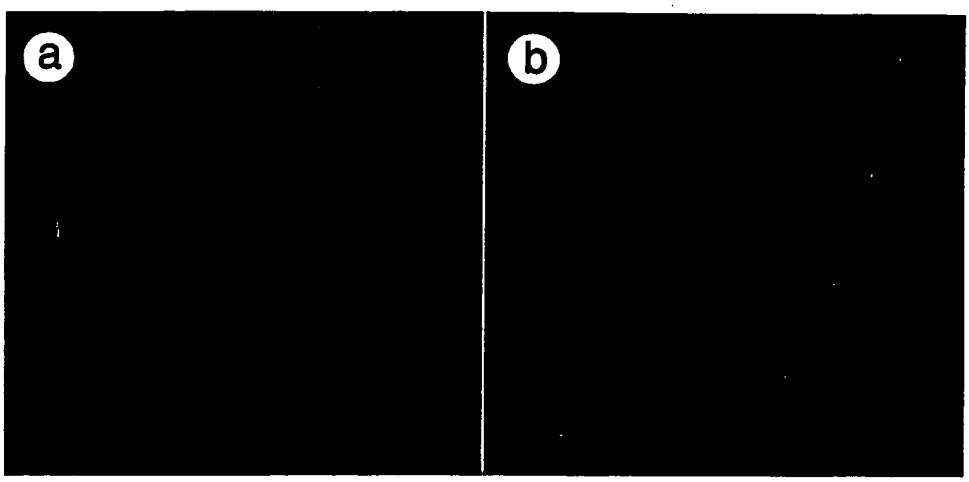


FIG. 3a FIG. 3b

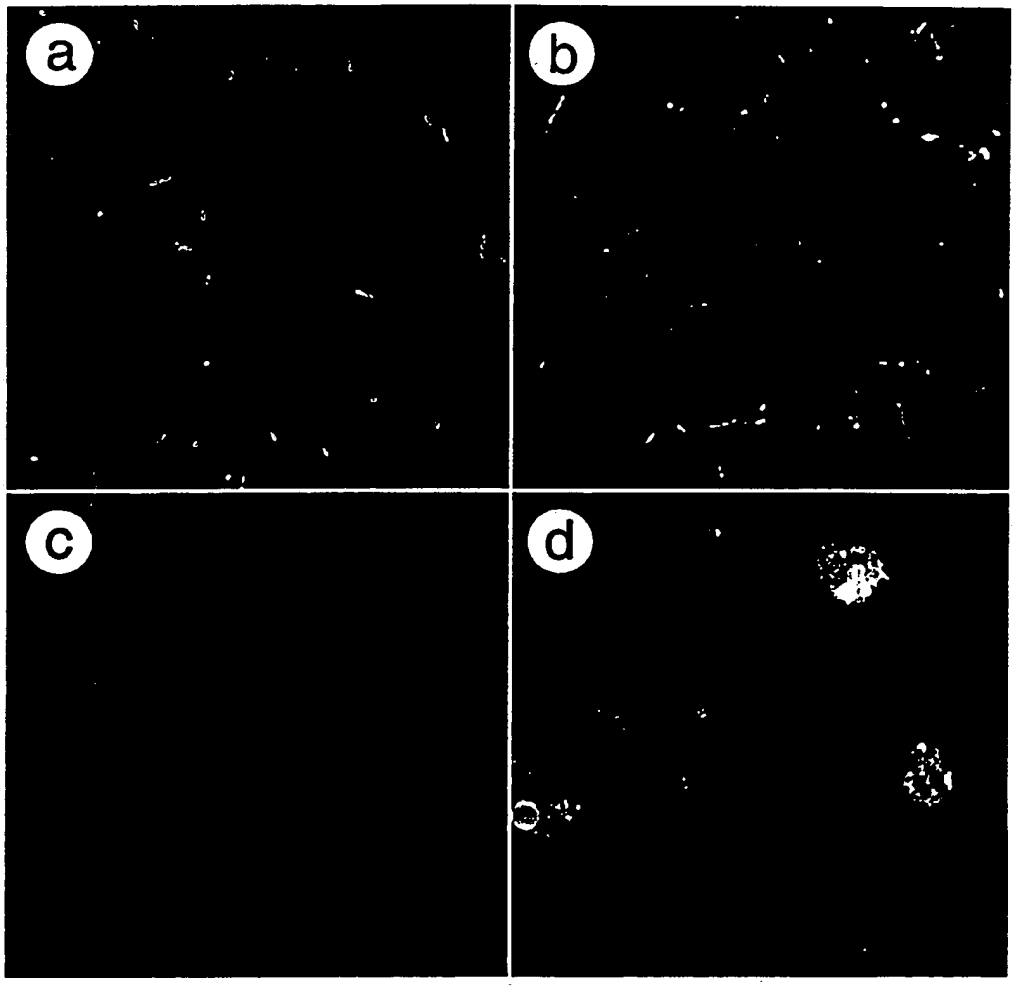


FIG. 4c

FIG. 4d